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CALCITONIN GENE-RELATED PEPTIDE STIMULATES CYCLIC AMP FORMATION IN RAT AORTIC SMOOTH MUSCLE CELLS

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SUMMARY: In rat aortic smooth muscle cells in culture, calcitonin gene-related peptide stimulated cAMP formation in a dose-dependent manner, half-maximally effective at 0.5 to 1 nM. There was no effect on formation of cGMP, which was increased 300-fold in the same experiments by atriopeptin or sodium nitroprusside. The vasodilator effect of CGRP in rat aorta requires an intact endothelium, indicating that increase in vascular smooth muscle cAMP is not in itself sufficient to bring about relaxation. cAMP is probably a mediator of CGRP action in vascular smooth muscle.

Calcitonin gene-related peptide (CGRP) is located widely in the central and peripheral nervous systems (1). The existence of this peptide was predicted (2) from analysis of rat calcitonin cDNA, in which proteolytic cleavage sites were found flanking a 37 amino acid region in the carboxy-terminal portion of the calcitonin gene transcript. Recent observations suggest that calcitonin and CGRP have discrete binding sites in the brain and spinal cord (3). Studies of membrane adenylate cyclase responses in several tissues possessing calcitonin and CGRP receptors indicated that CGRP had no independent stimulatory effect on adenylate cyclase, but that it behaved as a weak calcitonin-like agonist in some calcitonin-responsive adenylate cyclase systems (4).

CGRP has recently been shown to exert a potent vasodilator effect on rat aorta and in the cutaneous microvasculature of rabbit and man (5). The present experiments were carried out to determine whether the peptide had any direct effects on cyclic nucleotide metabolism in vascular smooth muscle cells. It is revealed as a potent activator of adenylate cyclase in cultured rat aortic smooth muscle cells, but the presence of endothelium is also required for CGRP to induce relaxation of rat aorta in vitro.

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MATERIALS AND METHODS

Chemicals and Hormones

Culture media and fetal calf serum were obtained from Flow Laboratories, Mt. Waverley, Australia. Synthetic human and rat CGRP were purchased from Bachem, Palo Alto, CA, USA, and synthetic salmon calcitonin was provided by the Armour Pharmaceutical Company, Kankakee, Illinois. Synthetic human parathyroid hormone (1-34) was obtained from Beckman Pty. Ltd., Palo Alto, CA, and Arg-Arg-atriopeptin III was a gift to Professor A.E. Doyle from Dr E.H. Blaine, (Merck, Sharp and Dohme, Research Laboratories, West Point, Pa). Collagenase (type V, 410 U/mg) was obtained from Sigma Chemical Co., St Louis, MO, as was elastase (type III). [^{3}H]-adenine, [^{32}P]-cAMP, [^{125}I]-cAMP tyrosine methyl ester and [^{125}I]-cGMP tyrosine methyl ester were purchased from the Radiochemical Center, Amersham, U.K. Antisera to cAMP and cGMP were prepared in rabbits, and provided by Dr N.H. Hunt (Canberra, Australia). Cyclic AMP, cyclic GMP and other nucleotides were from Boehringer Mannheim, Australia, and all other chemicals were of reagent grade from standard suppliers.

Rat Smooth Muscle Cell Culture

Primary cultures were established from aortae of six week old Sprague Dawley rats using a slight modification of a method developed for pig aortic smooth muscle cells (6). The modifications were that aortae were incubated in collagenase at 37°C for 20 mins before stripping adventitiae, the full thickness muscle layers were used, and the culture medium used was Dulbecco's Modified Eagles' Medium in 5% fetal calf serum.

Hormone Effects on Cyclic Nucleotide Production

Replicate rat smooth muscle cultures were plated out into 9.5 cm^{2} multiwell dishes (Costar) and the effects of treatment upon cyclic nucleotide formation studied when cultures were confluent. In some experiments [^{3}H]-cAMP generation in response to treatment was measured after prelabelling cell ATP pools by 2 hr incubation with [^{3}H]-adenine (1-2 µCi/ml), exactly as previously described (7,8). Alternatively, cAMP and cGMP generation in rat smooth muscle cell cultures were assayed by specific radioimmunoassays for the nucleotides. After treatment of cells for 10 mins at 37°C in 9.5 cm^{2} culture wells in the presence of 1 mM isobutylmethylxanthine, 1 ml 95% ethanol pH 3.0 at 4°C was added to each well. After standing for 2 hr the acid-ethanol was removed and dried in a vacuum centrifuge. Samples were reconstituted for assay, acetylated and assayed as previously described (9,10). Specificities of the antisera have been reported (10).

Relaxation of Rat Aorta

Female Sprague-Dawley rats (200-250 g) were heparinized (250 i.u., i.v.) and killed by a blow to the head. The thoracic aorta was removed rapidly, cleared of fat and connective tissue, and cut into rings 3 mm wide, care being taken not to damage the endothelium. The rings were mounted on wires in 5 ml organ baths containing oxygenated Krebs-Henseleit solution, under a resting tension of 2.0 g. Developed tension in the rings was measured using Grass FTO3C strain gauge transducers. After a 60 min equilibration period, rings were contracted to between 60 and 90% of maximum by norepinephrine (0.01 to 0.1 µM) or phenylephrine (0.1 µM). Vasodilator agents were added in cumulative concentrations once maximum contractions were attained. In some rings, the endothelium was deliberately removed by gentle abrasion of the intimal surface with a moistened filter paper taper. Relaxations were expressed as percentages of the increases in tension attained before addition of the vasodilators.

RESULTS

CGRP stimulated [^{3}H]-cAMP formation in a dose-dependent manner in rat aortic smooth muscle cells prelabelled by incubation with [^{3}H]-adenine (Fig. 1). This was a

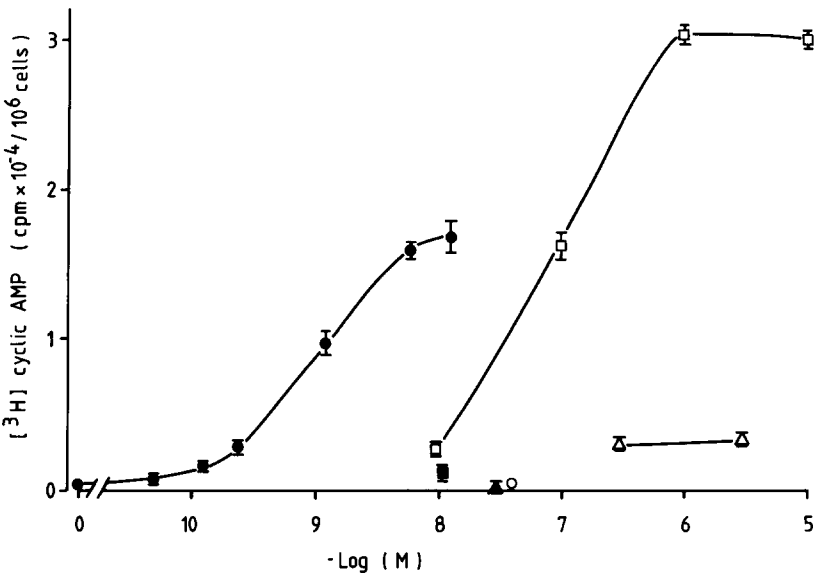

Figure 1 **Effects of CGRP (O) and isoproterenol (□) on [^{3}H]-cyclic AMP generation from [^{3}H]-ATP in rat aortic smooth muscle cells.**

Other symbols: PGE_2 (Δ), hPTH(1-34) (■), salmon calcitonin (▲), arg-arg-atriopeptin III (O). For details see Materials and Methods.

sensitive response and highly reproducible, with half-maximal effect of concentrations of CGRP ranging from 0.5 to 1 nM in several experiments. The amplitude of the response to CGRP was not as great as that to the β-adrenoreceptor agonist, isoproterenol, but was greater than that to prostaglandin E_2 or to prostacyclin (data not shown). Neither salmon calcitonin nor parathyroid hormone had any influence on cyclic AMP generation in the cells.

Since some agents which relax vascular smooth muscle stimulate cGMP formation, experiments were carried out to determine whether CGRP affected formation of this nucleotide. In these experiments radioimmunoassay for both cAMP and cGMP were carried out. The results (Fig. 2a and b) show again the effect of CGRP and isoproterenol on cAMP formation, with no effect of either atriopeptin or sodium nitroprusside. Neither CGRP nor isoproterenol had any effect on cGMP formation in the cells in these experiments, but atriopeptin increased cGMP formation by about 300-fold, and a substantial effect was seen with sodium nitroprusside as expected. Atriopeptin has been shown previously to stimulate guanylate cyclase in several rat tissues and to increase cGMP formation in rat aorta (11).

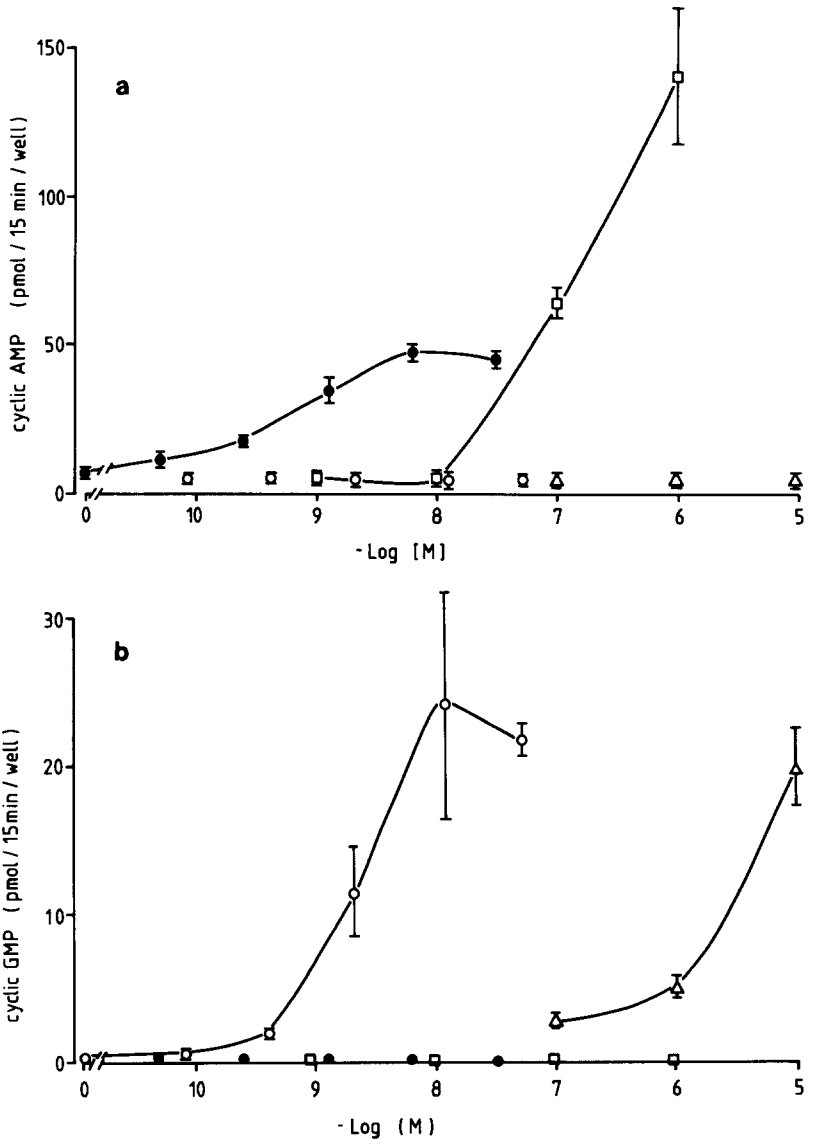

Figure 2 **Cyclic nucleotide production in rat aortic smooth muscle cells**

(a) Cyclic AMP: CGRP (●), isoproterenol (□), arg-arg-atriopeptin III (○), Na nitroprusside (Δ).
(b) Cyclic GMP. Same symbols.
For details see Materials and Methods.

CGRP caused weak, but dose-dependent relaxation of precontracted rat aortic rings but did not do so in rings that were denuded of endothelium (Fig. 3a). The presence of functional endothelium in rings was confirmed by marked relaxation to the calcium ionophore (A23187), which also was abolished in rings denuded of endothelium. However, in such rings that did not relax with CGRP or A23187, nitroprusside (1 μM) was still able to cause 100% relaxation (Fig. 3b), independent of the endothelium.

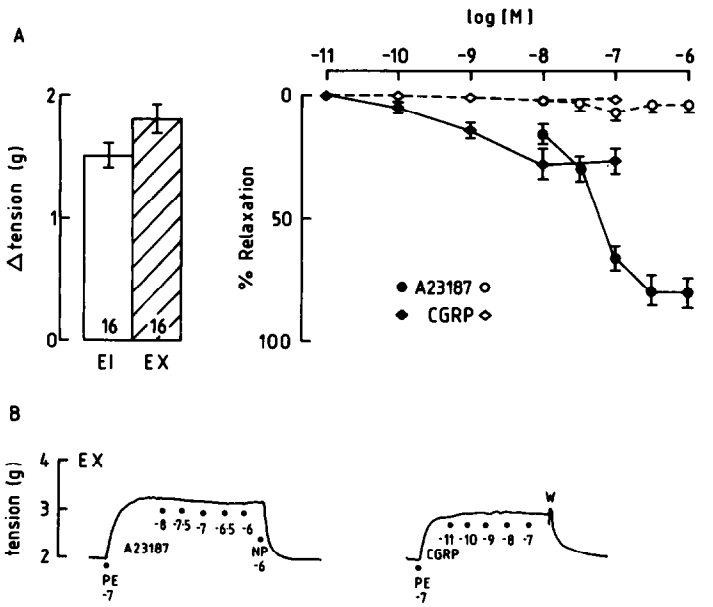

Figure 3A **Relaxation of rat aorta by CGRP and the calcium ionophore (A23187)**

The histograms represent the initial contractions produced by noradrenaline (0.1 μM) in 16 aortic rings with intact endothelium (EI) and 16 rings with disrupted endothelium (EX). The dose response curves are relaxations produced in 8 EI rings (solid lines) and 8 EX rings (broken lines). Vertical bars are standard errors of the means.

Figure 3B **Typical record of an aortic ring with disrupted endothelium (EX) that is precontracted with phenylephrine (PE) and does not relax with A23187 or CGRP, but is maximnally relaxed by nitroprusside (NP)**

The numbers represent molar concentrations.

DISCUSSION

These observations point to a sensitive, dose-dependent effect of CGRP on cAMP formation in rat aortic smooth muscle cells. This must reflect activation of adenylate cyclase by the peptide, since the experiments were carried out under conditions of maximum inhibition of phosphodiesterase. Although CGRP was not found to activate adenylate cyclase in brain and spinal cord membranes possessing CGRP receptors (4), leading to the suggestion that cAMP does not mediate CGRP action, the present data indicates that at least in vascular smooth muscle, CGRP might employ cAMP as a second messenger. The cGMP response was investigated because of the relationship between cGMP and smooth muscle relaxation (12). Sodium nitroprusside and atriopeptin were used as positive controls, with the latter producing a very substantial increase in cGMP formation, but CGRP was without effect.

The roles of cAMP and cGMP in vascular smooth muscle relaxation are not clearly defined. There is more evidence for cGMP than for cAMP as a critical second messenger in vascular dilatation (13). CGRP has a powerful vasodilator action in cutaneous vasculature (5) and in the present work we confirm that its effect in rat aorta (5) is entirely dependent on the presence of the endothelium. However, the CGRP-induced relaxation of rat aortic smooth muscle in the presence of endothelium is considerably less than that achieved by either the endothelium-dependent vasodilator A23187 or the endothelium-independent dilator, sodium nitroprusside. This could indicate that cGMP has a more direct role than cAMP in mediating rat aortic relaxation, and that elevation of cAMP alone in the smooth muscle is insufficient to trigger relaxation. Isoproterenol, on the other hand, does relax rat aortic smooth muscle in the absence of endothelium (data not shown); this might be explained by some other effect of isoproterenol, not shared by CGRP, or possibly by the greater cAMP response to isoproterenol. This will be investigated further.

Since rat aortic smooth muscle does not relax in direct response to CGRP, the cAMP response requires explanation. Its sensitivity, dose-dependency and amplitude are such that it seems most likely that it acts to mediate some action of CGRP on smooth muscle. This could be some as yet unknown response, or cAMP could contribute to the vasodilator effect of CGRP, which for more complete expression requires further input provided by the endothelium. The immunocytochemical location of CGRP in vascular nerve endings (14) raises the possibility that it may be an important factor in vascular function.

ACKNOWLEDGEMENTS

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